Molecular evolution: **Old branches on the phytochrome family tree**

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Phytochromes were thought to be unique to the plant world, but comparative genetic studies now show that they have evolved from an ancient family of light sensors with characteristics of prokaryotic two-component regulators.

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Current Biology 1998, 8:R117–R120
http://biomednet.com/elecref/09609822008R0117

Phytochromes are a divergent family of plant photoreceptors that mediate a wide range of developmental and physiological responses to changes in light intensity and spectral quality. In the flowering plants, phytochromes regulate seed germination, circadian rhythms, leaf and stem development, and the timing of floral initiation [1]. Each phytochrome molecule exists as one of two distinct photoreceptors: one form (called Pr) has an absorption maximum in the red region of the spectrum, while the other form (Pfr) has an absorption maximum in the far-red region near the end of the visible spectrum. Light absorption by Pr causes conversion to the Pfr form, and absorption by Pfr causes conversion back to the Pr form. The Pfr form is thought to be the active signaling molecule for most developmental and physiological responses.

Phytochromes are dimers, with each subunit consisting of a 1100–1200 amino-acid apoprotein covalently bound to a heme-derived, linear tetrapyrrole chromophore. The apoprotein is roughly divided into two major domains (Figure 1). An amino-terminal domain of ~670 amino acids contains the binding site for the chromophore, and a carboxy-terminal domain is needed for dimerization and is important in the transmission of light signals [1,2]. But, despite intensive study, the molecular mechanism of light perception by phytochromes, as well as the downstream signaling pathways, is essentially unknown.

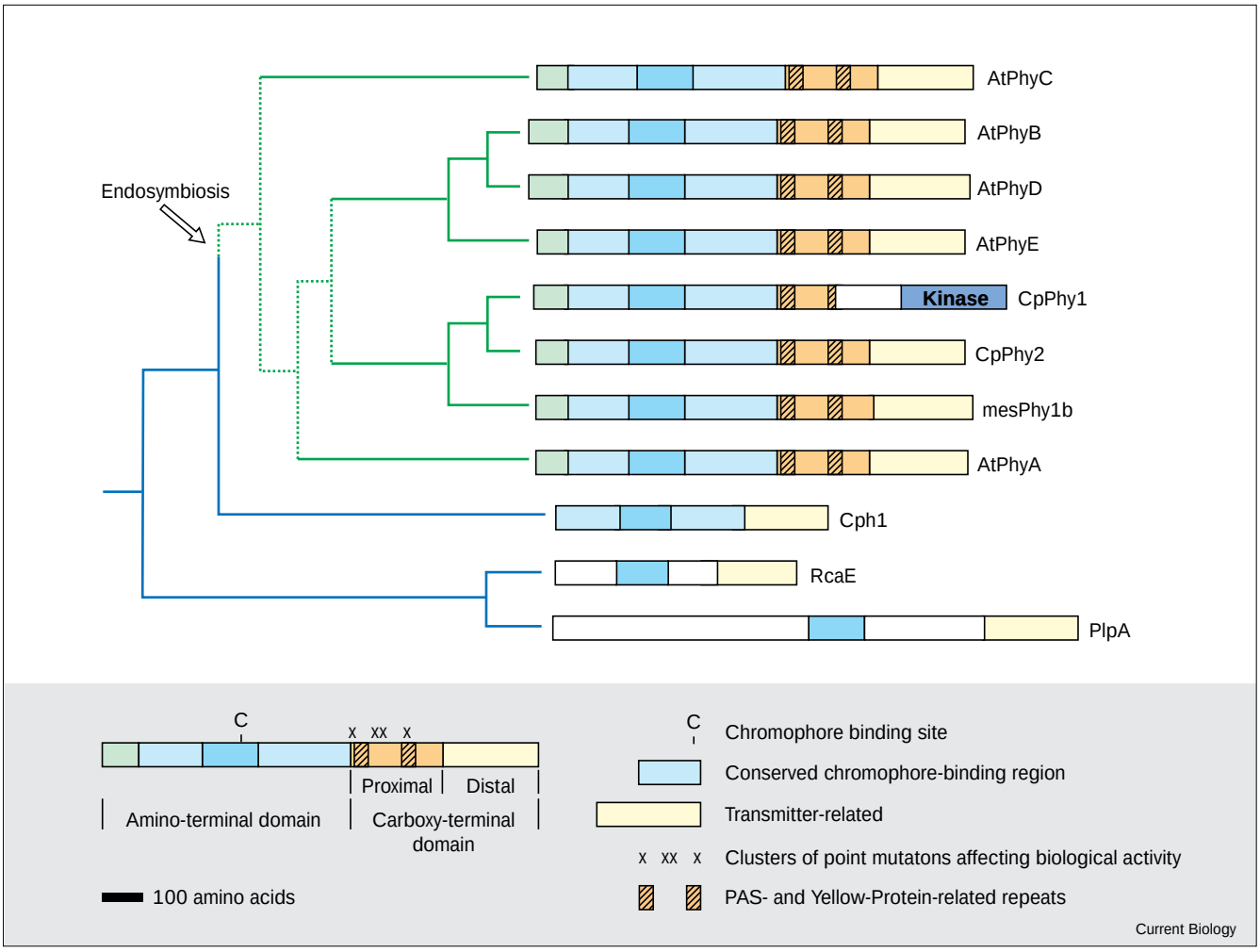
A clue to the mechanism of phytochrome action came with the discovery of amino-acid sequence similarity between a distal portion of the carboxy-terminal domain of plant phytochromes and 'transmitter' histidine kinase domains of eubacterial two-component regulatory systems [3]. These well-studied pathways perceive, transduce and often integrate signals from an astonishing array of environmental stimuli, including nutrients, toxins, osmolarity, temperature, acidity, redox state and light [4–6]. The molecular organization of these signaling systems is variable, but the most common pathway has two components: a sensor and a response-regulator [4].

A typical sensor polypeptide has a highly divergent amino-terminal 'input' domain that perceives the environmental signal, and a conserved carboxy-terminal 'transmitter' domain that has histidine autophosphorylation activity. Like plant phytochromes, sensor proteins are dimers, and dimeric structure may be essential to the sensing mechanism. A typical response-regulator protein also has two domains: a conserved amino-terminal 'receiver' domain and a divergent carboxy-terminal 'output' domain that is often a transcriptional regulator. In response to a signal from the input domain, the transmitter domain of the sensor autophosphorylates. The phosphate is then transferred to a conserved aspartate residue on the receiver domain of the response-regulator protein. Each of these regulatory domains is highly modular in nature, and novel signaling pathways have been created through the evolutionary exchange of domains.

One of the signals that prokaryotes respond to is light. The initial evidence for the existence of prokaryotic two-component photosensors with sequence similarity to phytochrome came from studies of a phenomenon known as 'complementary chromatic adaptation' [6]. This effect, found in some photosynthetic cyanobacteria, reflects a set of regulatory pathways for optimizing the photosynthetic light-harvesting apparatus to the spectral quality of the available light. Red light stimulates the expression of the red-light-absorbing chromoprotein phycocyanin, and green light induces expression of the green-light-absorbing protein phycoerythrin.

A cyanobacterial mutant deficient in red-light regulation of phycocyanin genes was isolated, and although the product of this gene — called *rcaC*, for *r*egulator of *c*hromatic *a*daptation — shows no sequence similarity to plant phytochromes, it does have strong sequence similarity to response-regulators [5]. The link to phytochromes came with the isolation of a mutant lacking proper photoregulation of both phycocyanins and phycoerythrins [6]. This mutant was found to be defective in a gene, designated *rcaE*, with a 655 amino-acid open reading frame. A portion of the amino-terminal domain of RcaE shows similarity to a conserved region surrounding the chromophore-binding site of eukaryotic phytochromes, while the carboxy-terminal domain has all of the hallmarks of a prokaryotic transmitter, as well as similarity to the distal carboxy-terminal domain of plant phytochromes [6].

Figure 1



Structure and evolution of representative phytochromes. Shown in the lower panel of the figure is a schematic of a typical flowering plant phytochrome, based on a composite of PhyA and PhyB data from a variety of species [1,2]. The upper panel shows a hypothetical evolutionary tree including selected phytochrome molecules. *Arabidopsis thaliana* (At) PhyA, PhyB, PhyC, PhyD and PhyE are representative of phytochromes from flowering plants. CpPhy1 and CpPhy2 are phytochromes from the moss *Ceratodon purpureus* and mesPhy1b is from the green alga *Mesotaenium caldariorum*. Nucleotides corresponding to a conserved chromophore-binding region (residues 224–393 of *A. thaliana* PhyE) [6] were aligned using ClustalX. The cladogram was obtained by a heuristic search with Phylogenetic Analysis Using Parsimony (PAUP3.1.1). RcaE and PlpA, which formed a distinct clade in all analyses, were used as an outgroup to root the tree. The branching order shown in stippled lines was not strongly supported by bootstrap analysis.

Another phytochrome-like gene, designated *plpA*, was identified from the cyanobacteria *Synechocystis* sp. PCC 6803 using very low stringency hybridization to a maize phytochrome probe [7]. *plpA* encodes a 1371 amino-acid protein that, like RcaE, has segments with recognizable similarity to both the conserved chromophore-binding region and to the transmitter-like distal carboxy-terminal sub-domain of plant phytochromes. *Synechocystis* sp. PCC 6803 does not undergo complementary chromatic adaptation, but does show long-term adaptive changes in the stoichiometry of photosystem I and photosystem II in response to spectral changes. Photosystem I absorbs more light in the blue region of the spectrum and, as an adaptation to blue light, photosystem I components become more abundant relative to those of photosystem II. *plpA* mutants, created by homologous gene replacement, grow normally in red and far-red light, but cannot grow in blue light unless supplemented with glucose. This apparent defect in blue-light-dependent photosynthesis suggests that PlpA may be a regulatory photosensor that directs appropriate expression of photosystem I and photosystem II genes during long-term adaptation to blue light.

The complete nucleotide sequence of the *Synechocystis* sp. PCC 6803 genome has recently revealed several additional

open reading frames with similarity to plant phytochromes [8]. In particular, an open reading frame — designated *cph1*, for *c*yanobacterial *ph*ytochrome — was found to encode a 748 amino-acid protein with significant similarity to a portion of the amino-terminal domain (36% identity and 60% similarity) and the distal carboxy-terminal domain (20% identity and 52% similarity) of plant phytochromes. Further biochemical and biophysical evidence supports the view that Cph1 forms a phytochrome-like chromoprotein.

One of the defining properties of plant phytochrome apoproteins is that they will autocatalytically attach themselves to linear tetrapyrroles to form the spectrally active chromoprotein [1,9]. A recombinant Cph1 fusion protein showed autocatalytic attachment to the linear tetrapyrrole phycocyanobilin (PCB) [9,10]. Furthermore, the Cph1–PCB chromoprotein had a red, far-red absorption spectrum similar to plant phytochrome–PCB chromoproteins. A mutant Cph1 lacking the carboxy-terminal domain retained chromophore-binding activity, producing a chromoprotein with spectral properties that were indistinguishable from the full-length protein, indicating that the amino-terminal domain of Cph1 constitutes a photosensory 'module' much like the amino-terminal domain of eukaryotic phytochromes [9]. Given that PCB is present in ample quantities in cyanobacterial cells, Cph1 probably forms a chromoprotein similar to plant phytochromes *in vivo*.

The carboxy-terminal domain of Cph1 has all of the conserved features of transmitter histidine kinases. To test for light-dependent autophosphorylation *in vitro* [9], a Cph1–PCB chromoprotein was treated with either red or far-red light to drive the photoequilibrium towards either the Pfr or Pr form, respectively. In the presence of ATP, both Pr and Pfr showed autophosphorylation at a residue that was base-stable and acid-labile, properties consistent with phosphorylation at a histidine residue. The Pr form autophosphorylated itself to significantly higher levels than the Pfr form, indicating that Pr is likely to be the active signaling species. This observation runs counter to the plant paradigm, in which Pfr is thought to be the phytochrome form that is active in signaling.

Ten base-pairs downstream from the stop codon of *cph1* is another open reading frame — designated *rcp1*, for *r*esponse regulator for *Cp*h1 — encoding a 147 amino-acid polypeptide with strong similarity to the receiver domain of proteins in the CheY superfamily of response-regulators [9]. The close physical linkage of *cph1* and *rcp1* suggests that the two genes are in the same operon and, by analogy to other prokaryotic operons, may have related functions. To test whether Cph1 and Rcp1 might form a sensor/response-regulator pair, biochemical interactions between the two purified components were examined *in vitro*.

Cph1–PCB was allowed to autophosphorylate in far-red light for 30 minutes, then either photoconverted to Pfr using red light or kept as Pr. The phosphorylated Pr form was able to efficiently undergo phosphotransfer to Rcp1, while the phosphorylated Pfr failed to transfer its phosphate to Rcp1. Thus, a light-dependent two-component signaling pathway was reconstructed *in vitro* using purified components. The physiological role of the Cph1/Rcp1 signaling pathway is not yet known — at this point, the molecular biology of cyanobacterial phytochrome signaling has out-paced the biology. However, experiments are under way in several laboratories to identify a phenotype for mutants in which *cph1* and *rcp1* have been deleted. Light responses that might be affected in these mutants include long-term photoadaptation, phototaxis and circadian rhythms.

The new data suggest that phytochromes, once thought to be unique to the plant world, have evolved from an ancient family of light sensors with the structural and functional characteristics of prokaryotic two-component regulatory molecules. The similarity of Cph1 and plant phytochromes across the vast evolutionary distances that separate cyanobacteria and plants suggests that the ancestral phytochrome molecule evolved in a common ancestor of *Synechocystis* and the free-living progenitor of chloroplasts. After endosymbiosis, the phytochrome gene presumably entered the eukaryotic genome by lateral transfer from the chloroplast to the nucleus.

These discoveries have generated great excitement, but it is clear that there are major structural and functional differences between the cyanobacterial and plant phytochromes. First, the extreme amino-terminal region, essential for biological activity in plant phytochromes, is missing in Cph1 [9]. Second, the histidine-kinase-like domains of several plant phytochromes (including PhyB, PhyD and PhyE of *Arabidopsis thaliana*) do not have the conserved histidine residue that is autophosphorylated in bacterial transmitters. Furthermore, site-directed mutations in the oat PhyA gene that eliminate this histidine residue and other residues conserved among all histidine kinases did not reduce biological activity [11]. Finally, point mutations in plant phytochromes that do reduce biological activity (but do not affect protein stability, dimerization or chromophore binding) tend to cluster in the proximal carboxy-terminal sub-domain, which has no counterpart in the Cph1 molecule [2].

Indeed, the genetic evidence indicates that the acquisition of the proximal carboxy-terminal sub-domain was an important event in the evolution of plant phytochromes. This region contains a pair of 43 amino acid direct repeats with similarity to the PAS repeats of the *period* clock gene product of *Drosophila* [12], which may be involved in protein–protein interactions. This repeat also shows

similarity to Yellow Protein, a chromoprotein photoreceptor found in the purple photosynthetic bacterium *Ectothiorodospira halophia*, and to segments of bacterial transmitters FixL and NtrY. The proximal sub-domain may indeed be another transmitter module 'wired' into the amino-terminal photosensory domain. Evidence that the intrinsic modularity of prokaryotic two-component regulators has been retained in plant phytochromes can be seen in the Phy1 protein of the moss *Ceratodon purpureus*, in which much of the carboxy-terminal domain has been replaced by a domain with similarity to a *Dictyostelium* tyrosine kinase and RAF serine/threonine kinases [13]. Perhaps one or both of the carboxy-terminal sub-domains of plant phytochromes mediate signal transmission by a different class of kinase [14].

Despite the differences between plant and bacterial phytochromes, the basic mechanisms of light perception and signal generation may be highly conserved. Unlike plant phytochromes, which are difficult to express in *Escherichia coli*, Cph1 can be produced in massive quantities for structural studies such as X-ray crystallography [10]. In the bacterial system, a wide range of mutant forms of Cph1 can be generated *in vitro* and purified for structural and biochemical analysis. These same mutant forms can be readily introduced into cyanobacteria by gene replacement and tested for *in vivo* function. Furthermore, as the biological role of the Cph1/Rcp1 pathway becomes better understood, genetic screens will be developed to identify all of the components in the cyanobacterial phytochrome signaling pathway. The application of this powerful combination of facile genetic and biochemical methodologies will profoundly improve our understanding of the mechanism of phytochrome action.

Acknowledgements

I wish to thank A. Jones, J.C. Lagarias and Y.M. Park for discussing results prior to publication. I wish to further thank S. Golden, T. McKnight, J. Manhart, M. Manson, P.H. Quail and J. Reed for helpful interactions.

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